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Application of a bacterial two-hybrid system for the analysis of protein–protein interactions between FemABX family proteins

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Protein–protein interactions play an important role in all cellular processes. The development of two-hybrid systems in yeast and bacteria allows for *in vivo* assessment of such interactions. Using a recently developed bacterial two-hybrid system, the interactions of the *Staphylococcus aureus* proteins FemA, FemB and FmhB, members of the FemABX protein family, which is involved in peptidoglycan biosynthesis and β-lactam resistance of numerous Gram-positive bacteria, were analysed. While FmhB is involved in the addition of glycine 1 of the pentaglycine interpeptide of *S. aureus* peptidoglycan, FemA and FemB are specific for glycines 2/3 and 4/5, respectively. FemA–FemA, FemA–FemB and FemB–FemB interactions were found, while FmhB exists solely as a monomer. Interactions detected by the bacterial two-hybrid system were confirmed using the glutathione *S*-transferase-pulldown assay and gel filtration.

INTRODUCTION

Protein–protein interactions play an important role in virtually all cellular processes. The yeast two-hybrid (YTH) system has become a widely used tool for determining such interactions *in vivo* [for review, see Phizicky & Fields (1995)]. With the complete sequencing of many prokaryotic and eukaryotic genomes, it has even become possible to develop proteome-wide interaction maps using high-throughput YTH systems. In prokaryotes, this has so far been done for *Helicobacter pylori* (Rain *et al.*, 2001). In *Staphylococcus aureus*, interactions between FtsA and FtsZ have been analysed using the conventional YTH system (Yan *et al.*, 2000, 2001), as have interactions between MecI and BlaI (McKinney *et al.*, 2001).

With the development of bacterial two-hybrid (BTH) systems (Dove *et al.*, 1997; Karimova *et al.*, 1998), bacterial proteins can now be assayed for interaction under conditions that match their native environment more closely. In the system developed by Karimova *et al.* (1998) (Fig. 1), the interaction of two proteins fused to *Bordetella pertussis* adenylate cyclase domains leads to its functional reconstitution and activation of subordinate metabolic pathways, allowing growth on minimal media or assaying for colour formation on MacConkey agar. This BTH system circumvents some of the limitations of the conventional YTH system, in which proteins localized in membranes and transcription factors, for example, are not amenable for analysis. In addition, the spatial separation of the interaction event and the signal readout reduces problems arising from non-specific interactions (Karimova *et al.*, 1998).

To validate the BTH system for use with *S. aureus* proteins, we have screened for interactions between FemA, FemB and FmhB, members of the FemABX family of peptidyl transferases that is centrally involved in β-lactam resistance

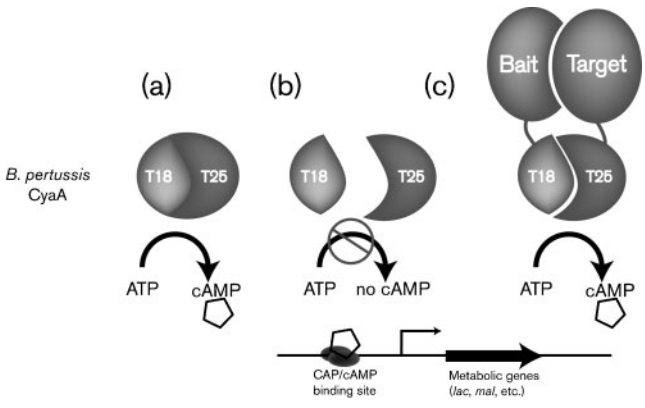

Fig. 1. BTH system (adapted from Karimova *et al.*, 1998). The *B. pertussis* CyaA protein consists of two functional domains, T18 (amino acids 1–224) and T25 (amino acids 225–399) (a). As the protein is split into its domains, it is no longer able to synthesize cAMP (b). Brought into spatial proximity by interaction of a 'bait' and 'target' protein, cAMP synthesis is enabled (c), activating CAP (catabolite activator protein)/cAMP-dependent metabolic genes such as the *lac* and *mal* operons.

Abbreviations: BTH, bacterial two-hybrid; GST, glutathione *S*-transferase; His_6, hexahistidine; HRP, horseradish peroxidase.

and the formation of branched muropeptides in a number of Gram-positive organisms. In *S. aureus*, these genetic factors have a dramatic impact on viability and resistance to methicillin and other antimicrobial substances (reviewed by Rohrer & Berger-Bächi, 2003).

We show here that homodimerization occurs for FemA and FemB, but not for FmhB, and that an interaction also occurs between FemA and FemB. The findings of the BTH screen were confirmed using two independent methods, namely, analytical gel filtration and the glutathione *S*-transferase (GST)-pulldown assay.

METHODS

Plasmid construction. Strains and plasmids used in this work are listed in Table 1. Molecular biology manipulations were done following standard procedures (Ausubel *et al.*, 1997). The 'bait'/'prey' vectors pKT25 and pUT18C, the control plasmids pKT25-zip and pUT18C-zip, and the reporter strain *Escherichia coli* DHM1 were a kind gift of D. Ladant (Institut Pasteur, France). *femA*, *femB* and *fmhB* were amplified from *S. aureus* BB255 genomic DNA by PCR and fused in-frame to the 3′ end of the *cyaA* gene fragments in plasmids pKT25 and pUT18C, respectively. C-terminal hexahistidine (His_6) tag fusions of FemA, FemB and FmhB were cloned in pET24b (Novagen). N-terminal GST fusions of FemA and FemB were cloned in pGEX-2T (Amersham Biosciences). PCR primers used are listed in Table 2.

Screening procedure. All combinations of the constructs were cotransformed into the reporter strain *E. coli* DHM1 (*cya*) on Luria–Bertani agar containing 100 mg ampicillin l^{-1} and 20 mg kanamycin l^{-1}. Positive controls (provided by D. Ladant) were GCN4 leucine zipper motifs cloned into pKT25 and pUT18C. Negative controls were the empty 'bait' and 'prey' vectors pKT25 and pUT18C. The cotransformants were spotted onto MacConkey agar containing 100 mg ampicillin l^{-1} and 20 mg kanamycin l^{-1}, and incubated at 30 °C for 2–3 days. Several colonies of each cotransformation were spotted to exclude clone-by-clone variation. Alternatively, clones were streaked onto M63 minimal agar containing ampicillin and kanamycin, and lactose as the sole carbon source. M63 agar was prepared using 15 g Bacto Agar l^{-1} (Difco), 2 g $(NH_4)_2SO_4$ l^{-1}, 13·6 g KH_2PO_4 l^{-1}, 0·5 mg $FeSO_4.7H_2O$ l^{-1}, adjusted to pH 7·0 with KOH. After autoclaving, 1 ml of 1 M $MgSO_4.7H_2O$, 15 ml of 20 % (w/v) lactose and 2 ml of 0·05 % (w/v) thiamin (all filter-sterilized) were added.

Production of recombinant protein. His_6-tagged protein was expressed in *E. coli* BL21(DE3) cells at 30 °C induced with 0·5 mM IPTG. Cells were lysed in 1/25 of the original culture volume of lysis buffer (50 mM sodium phosphate pH 8·0/150 mM NaCl/10 mM imidazole/1 mg lysozyme ml^{-1}/0·1 mg DNase ml^{-1}/0·01 mg RNase ml^{-1}/0·1 mM PMSF) on ice for 30 min. The lysates were then sonicated and centrifuged at 20 000 ***g*** for 20 min.

Cleared lysates were incubated with 1/20 volume of Ni-NTA–agarose (Qiagen) for 1 h, on an overhead shaker, at 4 °C. The resin was washed with 10–15 volumes of wash buffer (50 mM sodium phosphate pH 8·0/150 mM NaCl/10 mM imidazole). Protein was eluted with 3–4 column volumes of elution buffer (50 mM sodium phosphate pH 8·0/100 mM NaCl/200 mM imidazole). The eluates were centrifuged in Centricon centrifugal filter devices (Millipore)

Table 1. Strains and plasmids used in this work

Strain/plasmid	Genotype/description*	Origin/reference
Strain		
S. aureus BB255	Essentially as for NCTC 8325; template for PCR	Berger-Bächi (1983)
E. coli DH5α	Cloning strain	Invitrogen
E. coli BL21(DE3)	Expression strain	Novagen
E. coli DHM1	Reporter strain for BTH system; *cya*	D. Ladant; Karimova *et al.* (1998)
Plasmid		
pKT25	Two-hybrid plasmid, *cyaA*T25 fusion; Kan^r	D. Ladant; Karimova *et al.* (1998)
pUT18C	Two-hybrid plasmid, *cyaA*T18 fusion; Amp^r	D. Ladant; Karimova *et al.* (1998)
pKT25-zip	Two-hybrid control plasmid	D. Ladant
pUT18C-zip	Two-hybrid control plasmid	D. Ladant
pET24b	Expression plasmid for His_6 fusions; Kan^r	Novagen
pGEX-2T	Expression plasmid for GST fusions; Amp^r	Amersham Biosciences
pKT25*fmhB*	Two-hybrid plasmid containing *cyaAT25–fmhB* fusion	This work
pKT25*femA*	Two-hybrid plasmid containing *cyaAT25–femA* fusion	This work
pKT25*femB*	Two-hybrid plasmid containing *cyaAT25–femB* fusion	This work
pUT18C*fmhB*	Two-hybrid plasmid containing *cyaAT18–fmhB* fusion	This work
pUT18C*femA*	Two-hybrid plasmid containing *cyaAT18–femA* fusion	This work
pUT18C*femB*	Two-hybrid plasmid containing *cyaAT18–femB* fusion	This work
pET24b*fmhB*	Expression plasmid to produce FmhB with His_6 fused to C terminus	This work
pET24b*femA*	Expression plasmid to produce FemA with His_6 fused to C terminus	This work
pET24b*femB*	Expression plasmid to produce FemB with His_6 fused to C terminus	This work
pGEX2T*femA*	Expression plasmid to produce FemA with GST fused to N terminus	This work
pGEX2T*femB*	Expression plasmid to produce FemB with GST fused to N terminus	This work

*Kan^r, kanamycin-resistant; Amp^r, ampicillin-resistant.

Table 2. Oligonucleotides used in this work

Primer designation	Oligonucleotide sequence	Restriction site
femA BTH pKT25 fwd	GAACTGCAGTGAAGTTTACAAATTTAACAG	*Pst*I
femA BTH pUT18C fwd	GATCTGCAGGAAGTTTACAAATTTAACAG	*Pst*I
femA BTH rev	GCAGGTACCCTAAAAAATTCTGTCTTTAAC	*Kpn*I
femB BTH pUT18C fwd	GCACTGCAGGAAATTTACAGAGTTAACTG	*Pst*I
femB BTH pKT25 fwd	GCACTGCAGTGAAATTTACAGAGTTAACTG	*Pst*I
femB BTH rev	GCAGGTACCTTAATTTTTTACGTAATTTATC	*Kpn*I
fmhB BTH pUT18C fwd	CTACTGCAGGGAAAAGATGCATATCAC	*Pst*I
fmhB BTH pKT25 fwd	CATCTGCAGTGGAAAAGATGCATATCAC	*Pst*I
fmhB BTH rev	GCAGGTACCTATTTTCGTTTTAATTTACG	*Kpn*I
fmhB pET24b fwd	CGAGCTAGCGAAAAGATGCATATCACTAATC	*Nhe*I
fmhB pET24b rev	GCACTCGAGTTTTCGTTTTAATTTACG	*Xho*I
femA pET24b fwd	CGAGCTAGCAAGTTTACAAATTTAACAGC	*Nhe*I
femA pET24b rev	GCACTCGAGAAAAATTCTGTCTTTAAC	*Xho*I
femB pET24b fwd	CGAGCTAGCAAATTTACAGAGTTAACTGTTAC	*Nhe*I
femB pET24b rev	GCACTCGAGTTTCTTTAATTTTTTACTAATTTATC	*Xho*I
femA pGEX fwd	GTTGGATCCAAGTTTACAAATTTAACAGCTA	*Bam*HI
femA pGEX rev	GTTGAATTCCTAAAAAATTCTGTCTTTAACTTT	*Eco*RI
femB pGEX fwd	CAAGGATCCAAATTTACAGAGTTAACTGTTAC	*Bam*HI
femB pGEX rev	GTTGAATTCTTTAATTTTTTACGTAATTTATCC	*Eco*RI

with a cut-off of 10 kDa to exchange the buffer for PBS (8·5 g NaCl l^{-1}, 10·7 g $Na_2HPO_4.2H_2O$ l^{-1}, 0·9 g KH_2PO_4 l^{-1}, pH 7·4). The recombinant protein was more than 95 % pure on SDS-PAGE and Coomassie staining (not shown).

GST fusions of FemA and FemB were purified similarly. The fusion proteins were expressed in *E. coli* BL21(DE3) cells with 0·05 mM IPTG at 17 °C for 1 h. Cells were lysed in 1/25 of the original culture volume of lysis buffer as above, with addition of 1 % (v/v) Triton X-100, omitting imidazole. Cleared lysates were bound to 1/40 to 1/20 volume of glutathione–agarose (Sigma) at 4 °C for 1 h, on an overhead shaker, washed with 20 volumes of PBS and eluted with 50 mM Tris/HCl pH 8/150 mM NaCl/5 mM GSH. The protein was more than 95 % pure on SDS-PAGE and Coomassie staining (not shown).

Analytical gel filtration. Protein oligomerization was observed by analytical gel filtration using FPLC on a Superdex 200 HR column on an Äkta FPLC system (Pharmacia). Proteins were run in PBS. In addition to recombinant His_6-tagged proteins, a set of protein standards were run (HMW and LMW protein standards; Pharmacia). Peak data of the standards were used to perform linear regression analysis, in order to obtain a standard curve for molecular mass determination of the analysed proteins.

GST-pulldown assay. Cleared lysates of GST–FemA or GST–FemB fusions, or GST alone, were incubated with equal volumes of GSH–agarose beads for 1 h at 4 °C. The bead material was washed with 20 volumes of PBS. Non-specific protein binding was blocked by incubating the bead material for 1 h with BSA (1 mg ml^{-1}) in PBS at 4 °C. The material was again washed with 20 volumes of PBS. His_6-tagged FemA or FemB protein was added in excess and incubated with the bead-bound proteins for 2 h at 4 °C. Unbound protein was removed by washing three times with 10 volumes of PBS, and the bead samples with bound protein were suspended in equal volumes of SDS sample buffer.

Western blotting. SDS-PAGE and Western blotting were done following standard procedures (Ausubel *et al.*, 1997). Western blots were incubated with monoclonal anti-His antibody (Sigma) at 1 : 1000 dilution in PBS containing 0·03 % (w/v) Top Block (Juro) overnight at 4 °C, followed by secondary antibody (Sheep anti-mouse F_{ab}–HRP; Jackson Immuno Research) at 1 : 10 000 dilution for 2 h at room temperature (22 °C). Detection was done with SuperSignal West Femto reagent (Pierce).

RESULTS

BTH screening

fmhB, *femA* and *femB* were each cloned into the 'bait' and 'prey' vectors and all nine combinations were cotransformed into the reporter strain DHM1. Cotransformants were streaked onto MacConkey or M63 minimal agar plates containing lactose as the sole carbon source. After 2–3 days incubation at 30 °C, colour formation was observed for interactors on MacConkey agar, while non-interactors remained white (Fig. 2). The positive controls, two leucine-zipper domains, gave rise to stronger colour formation, which was assumed to be due to very strong interaction between these proteins. On minimal agar, positive clones grew after 3–4 days (not shown).

Positive clones were obtained for the combinations pUT18C*femA*/pKT25*femA*, pUT18C*femA*/pKT25*femB*, pUT18C*femB*/pKT25*femA* and pUT18C*femB*/pKT25*femB*, while all combinations with *fmhB* were negative. We therefore assumed an interaction for the positive combinations and proceeded to confirm these interactions using independent methods.

Analytical gel filtration

His_6-tagged protein was run on a Superdex 200 HR size exclusion column to determine protein oligomerization.

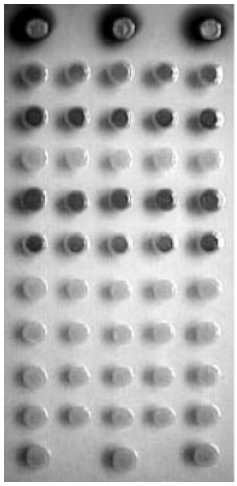

Fig. 2. Growth of FemAB/FmhB BTH cotransformants on MacConkey agar. Several clones of each 'bait'/'prey' plasmid cotransformation were spotted, along with positive and negative controls. *lac*-positive clones (interactors) show colour formation (*), while non-interactors remain white. Due to a very strong interaction, the positive control gives a strong signal.

A standard curve was derived by running protein standards under the same conditions and performing linear regression analysis on the peak data. From this standard curve, the peak sizes of the recombinant proteins could be determined. For the monomers of FmhB, FemA and FemB, the measured molecular masses correlated well with the theoretical values derived from the amino acid sequences, and it was determined that FemA and FemB formed dimers (Fig. 3a, b; Table 3).

GST-pulldown assay

Since by analytical gel filtration a FemA/FemB heterodimer was not expected to be distinguishable from their homodimers, GST-pulldown assays were done. FemA and FemB were expressed as GST fusions. To control for non-specific binding, GST alone was used. GST, GST–FemA or GST–FemB fusions bound to GSH–agarose were incubated with equal amounts of His_6-tagged FemA or FemB, respectively. After washing, bound protein was analysed by Western blotting using an anti-His_6 antibody (Fig. 3c). While a limited extent of non-specific binding to GST or GSH–agarose was observed for FemB, significant specific binding to GST–FemA and GST–FemB, respectively, was observed for both FemA and FemB, confirming their homo- and heterodimerization *in vitro* that had been observed in the BTH system.

DISCUSSION

We have demonstrated here that the BTH system developed by Karimova *et al.* (1998) can be applied to the analysis of staphylococcal proteins, by showing interaction of FemA–FemA, FemA–FemB and FemB–FemB. Although the interacting clones were always negative in combination with FmhB, which excluded a false-positive result, any interactions found in a two-hybrid assay have to be validated carefully using independent methods to rule out false-positives. Analytical gel filtration allowed us to confirm homodimerization of FemA and FemB, and demonstrated that FmhB is very likely a monomer *in vivo*, but we cannot rule out protein–protein interaction(s) mediated by some auxiliary molecule in *Staphylococcus*. The GST-pulldown assay was done to confirm unanimously all interactions found in the BTH screen.

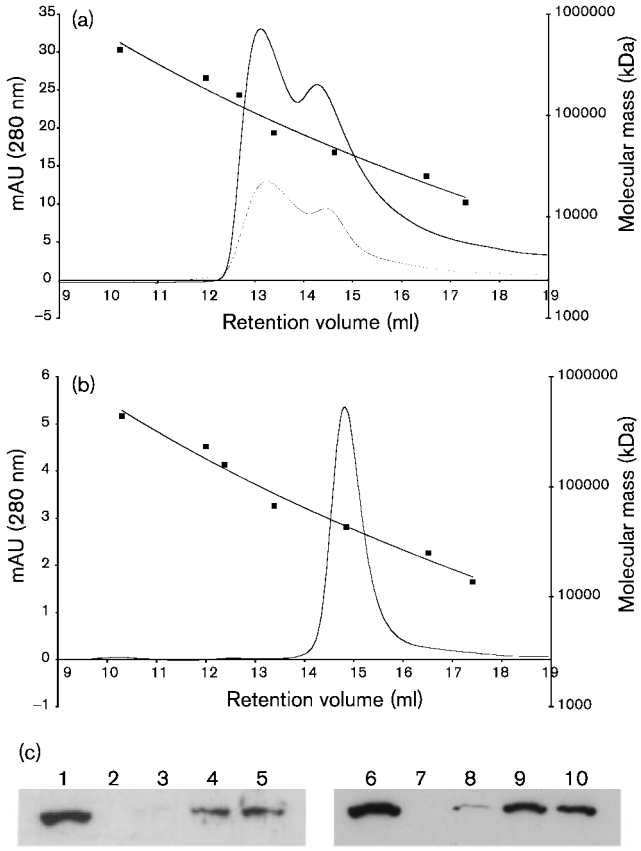

Fig. 3. Analytical gel filtration and GST-pulldown. (a) Gel filtration of FemA (thin line) and FemB (thick line); (b) gel filtration of FmhB. Standard curves were calculated using linear regression analysis. Peak sizes are listed in Table 3. mAU, Milliabsorbance units. (c) GST-pulldown assay analysed by Western blot. Equal amounts of sample were loaded. His_6-tagged protein was detected using monoclonal anti-His_6 antibody (Sigma) at 1 : 1000 dilution, followed by secondary antibody (Sheep anti-mouse F_{ab}–HRP; Jackson Immuno Research) at 1 : 10 000 dilution. Lanes: 1, His_6–FemA (reference lane); 2, blank; 3, GST control protein + His_6–FemA; 4, GST–FemA + His_6–FemA; 5, GST–FemB + His_6–FemA; 6, His_6–FemB (reference lane); 7, blank; 8, GST control + His_6–FemB; 9, GST–FemA + His_6–FemB; 10, GST–FemB + His_6–FemB.

Table 3. Molecular masses of FmhB, FemA and FemB as measured by gel filtration

Protein	Theoretical molecular mass (kDa)	Measured molecular mass (kDa)	Measured dimer molecular mass (kDa)
FmhB	48·5	50·0	NA
FemA	49·1	49·4	89·3
FemB	49·6	53·8	95·4

NA, Not applicable.

Additional BTH experiments were done using other members of the FemABX family. The lysostaphin immunity factor Lif protects *Staphylococcus simulans* biovar *staphylolyticus* from the action of lysostaphin and has been shown to substitute serine in positions 3 and 5 of the pentaglycine side chain in *S. aureus*, in cooperation with FemA and FemB (Ehlert *et al.*, 2000; Thumm & Götz, 1997). We observed interactions between Lif and both FemA and FemB, but not with FmhB, nor Lif homodimerization (data not shown). *Streptococcus pneumoniae* FibB (MurN) adds alanine or serine to the stem peptide in cooperation with FibA (MurM) to form an Ala_2 or Ala–Ser side chain (Filipe & Tomasz, 2000; Weber *et al.*, 2000). FibB proved to be able to form homodimers, but not heterodimers with FibA, and FibA homodimerization was also not observed (data not shown). To bring these additional findings into perspective, the sequences of known FemABX family proteins were compared (Fig. 4). It appears that all proteins able to form dimers group in one of two main clades of the family. The non-interactors are grouped in the other,

Fig. 4. Dendrogram of the FemABX protein family adapted from Rohrer & Berger-Bächi (2003). Protein sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994) and the dendrogram was constructed using TREEVIEW (Page, 1996). The labels of the numerous FemA homologues of staphylococci were enlarged, for ease of view. *S.*, *Staphylococcus*; *St.*, *Streptococcus*; *C.*, *Clostridium*; *T.*, *Treponema*; *B.*, *Borrelia*; *D.*, *Deinococcus*; *Str.*, *Streptomyces*; *W.*, *Weissella*; *E.*, *Enterococcus*; *S. anaerobius*, *S. aureus* subsp. *anaerobius*; *St. zooepidemicus*, *St. equi* subsp. *zooepidemicus*.

evolutionarily older, branch comprising those FemABX family members that add the first amino acid in an interpeptide to the peptidoglycan, and/or are the only family member in the respective species. This may indicate that a gain of function occurred during the diversification of the FemABX protein family. However, this hypothesis would require further experimental testing. Analysis of protein–protein interactions within the FemABX protein family may yield evidence concerning their function. While there must be a mechanism that determines whether one or two amino acids are attached by a FemABX protein, the data presented here do not imply that interaction or non-interaction contribute to this mechanism.

The protein–protein interactions we were able to show add to the more-detailed knowledge about the FemABX protein family that has been gained in recent years (Benson *et al.*, 2002; Bouhss *et al.*, 2002; reviewed by Rohrer & Berger-Bächi, 2003). Proteins of the FemABX family are a recognized target for the development of novel antimicrobial agents (Kopp *et al.*, 1996). It is therefore crucial to understand all aspects of their function in order to advance towards the development of new medicines.

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